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TCF12 overexpression as a poor prognostic factor in ovarian cancer

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ABSTRACT

Introduction: Ovarian cancer is a common malignant tumor that is severely harmful to human health, but the molecular mechanisms of ovarian cancer remain unclear. Transcription factor 12 (TCF12) is a member of the basic helix-loop-helix (bHLH) E protein family, which recognizes the E-box sequence and is responsible for cellular development and differentiation. A recent study has reported that TCF12 is highly expressed in some human cancers and may be correlated with clinicopathological factors, but there are few studies on its mechanism. There is no report on TCF12 in ovarian cancer.

Materials and methods: The expression profiles of TCF12 in human ovarian cancer patients and cells were detected by immunohistochemistry (IHC), real-time quantitative PCR (RT-qPCR) and Western blot; MTT, wound-healing and transwell migration assays, as well as flow cytometry, were used to investigate the biological functions of TCF12 in A2780 and SK-OV-3 ovarian cancer cell lines.

Results: This study reports for the first time that TCF12 is overexpressed in patients with ovarian cancer and that its high expression is associated with histological grade and metastasis. TCF12 downregulation using small interfering RNA (siRNA) inhibited ovarian cancer cell growth, migration, and invasion and promoted apoptosis.

Conclusion: The results suggest that TCF12 is a poor prognostic factor of ovarian cancer and that targeting TCF12 may be a new therapeutic strategy for ovarian cancer treatment.

1. Introduction

Ovarian cancer is one of the most common malignant tumors in female reproductive organs, and the incidence of ovarian cancer ranks third, only behind cervical and uterine cancer [1–3]. Epithelial cell carcinoma is the most common type of ovarian cancer, followed by malignant germ cell carcinoma. The mortality rate of ovarian epithelial cell carcinoma is the highest among all kinds of gynecological tumors, and this cancer poses a serious threat to women's lives. Due to the complex embryonic development, histological anatomy and endocrine function of the ovary and to atypical early symptoms, it is very difficult to distinguish the histological types of benign and malignant ovarian tumors before operation, and ovarian epithelial cell cancer is prone to metastasis and invasion [4–6]; thus, its early diagnosis is very difficult. Early detection of ovarian malignant tumors, especially epithelial cell cancer, is difficult because no methods of diagnosis or effective therapy are available yet, and its etiology remains unknown. Therefore, there is an urgent need to find methods of early diagnosis and effective therapy for ovarian cancer.

Transcription factor 12 (TCF12) is a member of the basic helix-loop-helix (bHLH) E protein family, which recognizes the E-box sequence CANNTG [7,8]. The protein encoded by this gene is expressed in many tissues and cells, including skeletal muscle, thymus, B cells and T cells, and may participate in regulating the expression of strain-specific genes through heterodimerization with other bHLH E-proteins. The gene has many different transcriptional splicing variants, but the nature of the full length variant has not yet been determined. TCF12 coding protein (also known as HTF4 or Heb) is a member of the helix-loop-helix (HLH) protein family [9,10]. HLH proteins are classified into seven categories according to their tissue distribution, responsiveness, and DNA binding specificity [11]. Class I proteins, encoded by TCF3 (E12, E47), TCF4 (E2-2) and TCF12 (HTF4), are also known as E proteins because of their DNA (E-box) binding ability. These proteins are widely expressed in many tissues and can form homologous or heterodimers. Class II proteins, including MyoD, myogenin and TWIST, can form heterodimers with class I members and display tissue-specific expression patterns. Class III proteins contain a leucine zipper domain and an HLH domain and include the Myc transcription factor family. Class IV includes a family of proteins such as Mad, Max and Mxi, which can form dimers with Myc. Class V proteins lack basic regions and therefore cannot

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Table 1
TCF12-targeted siRNA sequences.

siRNA ID		Sequence (5'-3')
TCF12_si1	Sense	GGCAGAACAAGCAGUACUAdTdT
	Antisense	UAGUACUGCUUGUUCUGCCdTdT
TCF12_si2	Sense	CAGUGAUAUACAUAGUUUAdTdT
	Antisense	UAAACUAUGUAUAUCACUGdTdT
TCF12_si3	Sense	GGCAGUCAUCCUUAGUCUAdTdT
	Antisense	UAGACUAAGGAUGACUGCCdTdT
TCF12_si4	Sense	GCACAGACCUGAACCAUAAdTdT
	Antisense	UUAUGGUUCAGGUCUGUGCdTdT
NC	Sense	UUCUCCGAACGUGUCACGUdTdT
	Antisense	ACGUGACACGUUCGGAGAAdTdT

directly bind to DNA. The members of Class I and Class II are dominant negative factors. Class VI members have a proline in their basic region. Class VII proteins are classified according to their HLH-PAS domains. These proteins include hypoxia inducible factor 1A and aromatic hydrocarbon receptors. TCF12 can form homologous oligomers or hetero-oligomers with myogenic proteins, E12 and ITF12, and with PTF-1, RUNX1T1 [12] and the TCF regulator ID1 [13,14].

TCF12 is responsible for cell development and differentiation and plays a key role in differentiation of lymphocytes and in the development of neural and mesenchymal tissues [12,15,16]. Several studies have indicated that TCF12 may function as regulator in some tumors, such as glioma [17], breast cancer [18], colon cancer [19,20], oral cancer [21] and gallbladder cancer [22] and have also reported that TCF12 is closely related to tumor metastasis and invasion [18–20]. However, there are few studies on the molecular mechanism of TCF12 in clinical tumors, and no report on TCF12 in ovarian cancer has been published yet.

2. Materials and methods

2.1. Clinical patient samples

Human tissue samples were selected from patients with ovarian cancer at our department from 2005 to 2010. Written informed consent was obtained from all patients, and this study was approved by the Ethics Committee of Affiliated Hospital of Nantong University. All tissues were fixed in formalin and embedded in paraffin. In total, 116 cases of paraffin-embedded tissue samples were used for immunohistochemical staining, and 27 cases of human fresh ovarian cancer tissues and matched normal ovary tissues were collected to assess gene expression levels using the RT-qPCR method.

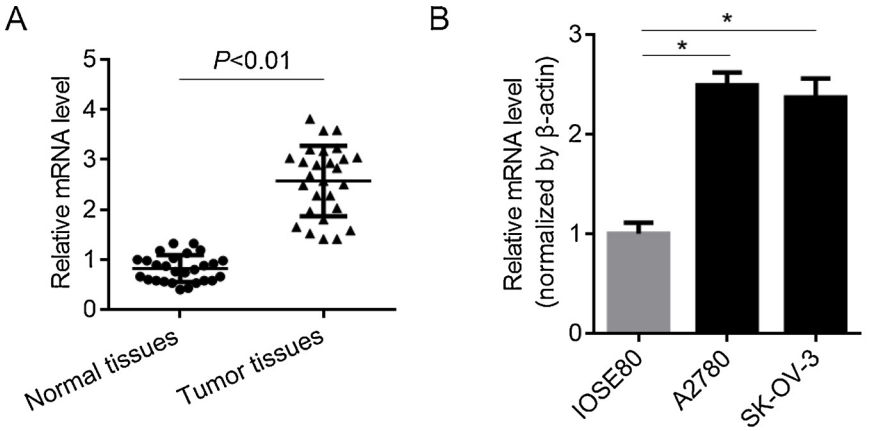
2.2. Cell culture and transfection

The human ovarian cancer cell lines A2780, SK-OV-3 and a normal ovarian epithelial cell line (IOSE80) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) at 37 °C in with 5% CO_2.

TCF12-targeting siRNAs were designed to inhibit TCF12 mRNA expression in ovarian cells, and the corresponding siRNA sequences are shown in Table 1. Non-human homology siRNA sequence used as negative control (NC). Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, USA) was used for siRNAs and NC transfection into cells according to the manufacturer's instructions. siRNAs and negative controls were purchased from Biomics Biotechnologies Co., Ltd. (China).

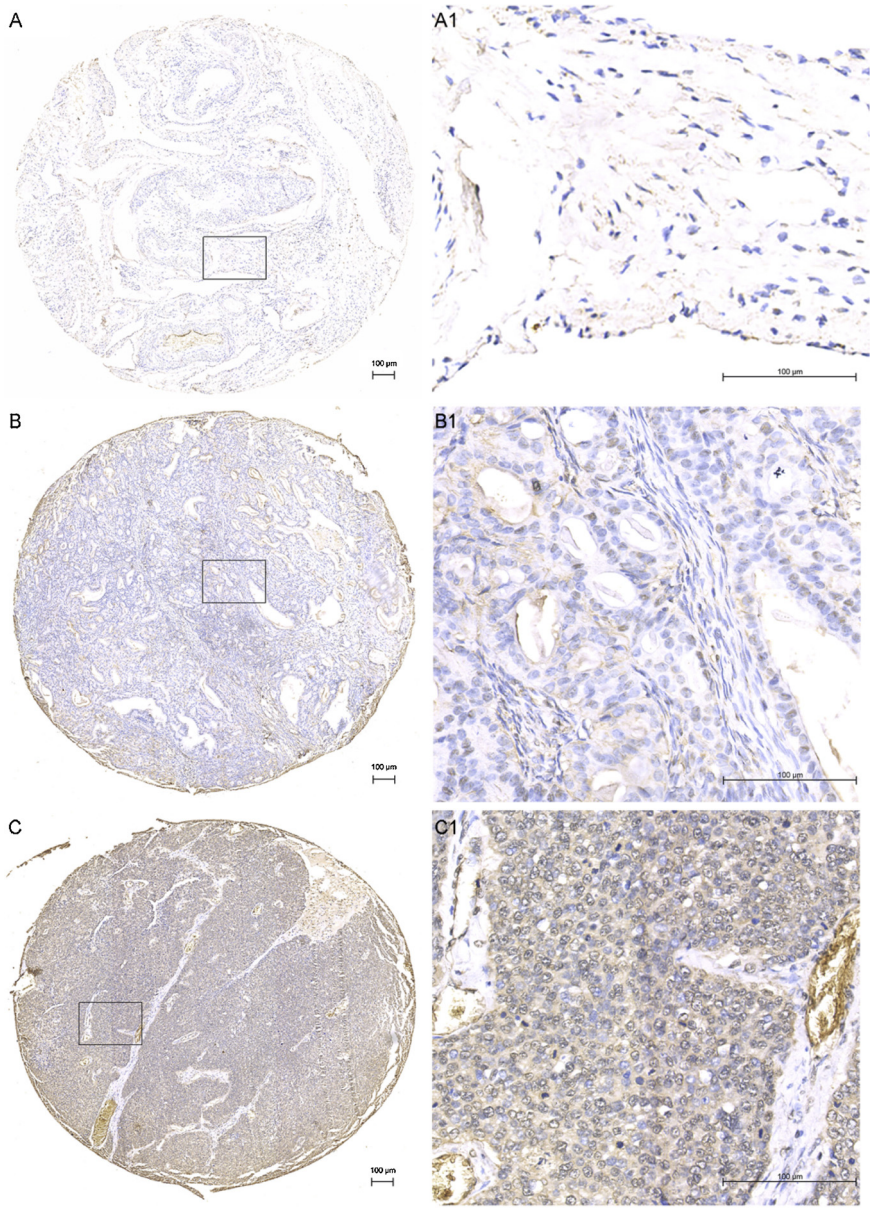
2.3. Immunohistochemical staining

The paraffin-embedded tissue samples were prepared as tissue microarray by the Department of Clinical Pathology, Affiliated Hospital of Nantong University (Nantong, China). The tissue microarray sections were determined by immunohistochemical staining using the Envision Plus/Horseradish Peroxidase system (DAKO, USA). The sections were incubated with a rabbit anti-human TCF12 primary antibody (1:200 dilution) (Abcam, USA) at 4 °C overnight. After being washed in phosphate-buffered saline (PBS), the sections were incubated with Envision Plus secondary antibody for 30 min, followed by diaminobenzidine solution for 5 min and counterstained with hematoxylin. Last, TCF12 staining intensity was evaluated and scored independently by two pathologists. Staining intensity was scored as 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). The percentage of positive cells was scored as 0 (≤5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) and 4 (> 75%). The scoring results were evaluated according to intensity and percentage scores. TCF12 expression was defined as: "−" (negative, score of 0), "+" (weakly positive, score of 1–4), "++" (positive, score of 5–8), "+++" (strongly positive, score of 9–12).



Abbreviations: TCF12, transcription factor 12; RT-qPCR, real-time quantitative PCR.

Fig. 1. TCF12 expression in ovarian cancer tissues and cells was detected by RT-qPCR. **A.** TCF12 expression in 27 cases of ovarian cancer tissues and paired normal tissues. **B.** TCF12 expression in ovarian cancer cell lines A2780 and SK-OV-3. *$P < 0.05$, compared with normal ovarian epithelial cell IOSE80.



Abbreviations: TCF12, transcription factor 12; IHC, immunohistochemical.

Fig. 2. TCF12 expression in ovarian cancer patients was analyzed by IHC staining. **A/A1**. Negative TCF12 staining in normal ovarian tissue (A, 5×; A1, 40×); **B/B1**. Weak TCF12 staining in cancer tissue (B, 5×; B1, 40×); **C/C1**. Strong TCF12 staining in cancer tissue (C, 5×; C1, 40×).

2.4. Real-time quantitative PCR (RT-qPCR)

After treatment for 48 h, as indicated above, total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, USA) according to the manufacturers' instructions. β-actin was used as an internal control. RT-qPCR reactions were carried out using the One-Step RT-qPCR kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method [23]. The following primers were used: TCF12 forward: 5′-CTAATGAAGATGAGGATT-3′; TCF12 reverse: 5′- GATGAAGAATAA GGAGTT-3′; β-actin forward: 5′-TTGCCGACAGGATGCAGAAGGA-3′; β-actin reverse: 5′-AGGTGGACAGCGAGGCCAGGAT-3′.

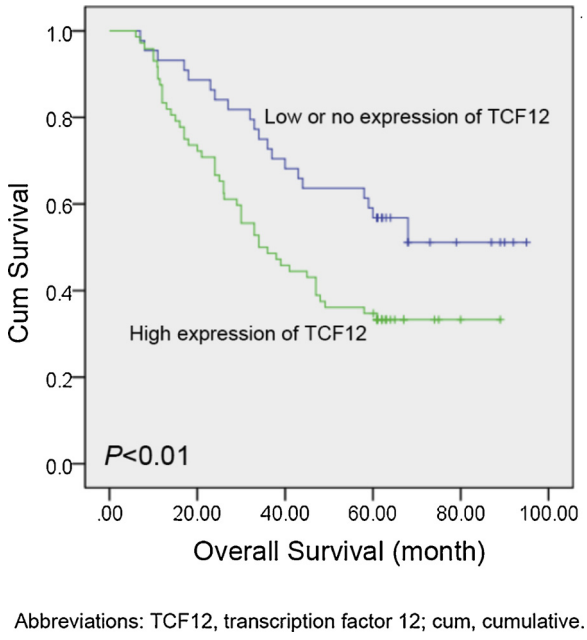
2.5. Western blot analysis

A total of 1×10^5 cells/well were plated into a 6-well plate and grown for 24 h. the Cells were treated for 48 h, as described above. After being washed in PBS, the cells were lysed in RIPA Buffer (Sigma-Aldrich, USA) on ice, transferred into centrifuge tubes, and boiled for 10 min. Total protein fractions were collected by centrifugation at 4 °C and at 12,000 rpm for 15 min, separated by SDS-PAGE, and transferred onto a PVDF membrane (GE, USA). Subsequently, the membrane was blocked in 5% evaporated milk for 2 h at room temperature and then incubated with the primary antibody, rabbit anti-human TCF12 antibody (1:500 dilution) (Abcam, USA) or with mouse anti-human β-actin antibody (1:1000 dilution) (Abcam, USA) at 4 °C overnight. After washing 3 times with TBST for 5 min, the PVDF membrane was incubated with the secondary antibody, horseradish peroxidase (HRP)-conjugated IgG (1:2000 dilution) (Abcam, USA) for 2 h at room temperature. After washing 3 times with TBST for 5 min, the specific proteins were detected with ECL Western Blotting Substrate (Promega, USA) and exposure using X-rays. The protein bands on X-rays were

Table 2
Association between TCF12 expression and clinical pathological factors in malignant ovarian cancer.

Clinicopathological parameters	No. Cases	TCF12 Expression, n		*Pearson* x^2	*P*
		-/+	+ +/+ + +		
Age				0.003	0.953
≤50	32	12	20		
> 50	84	32	52		
Pathological type				7.900	0.095
Serous carcinoma	79	25	54		
Mucinous carcinoma	5	2	3		
Endometrioid carcinoma	10	7	3		
Clear cell carcinoma	10	3	7		
Others	12	7	5		
FIGO stage				0.011	0.915
I/II	64	24	40		
III/IV	52	20	32		
Histological grade				5.425	0.020*
I/II	45	23	22		
III	71	21	50		
Metastasis				9.638	0.002*
No	50	27	23		
Yes	66	17	49		

* represents statistical significance, $P < 0.05$.



Abbreviations: TCF12, transcription factor 12; cum, cumulative.

Fig. 3. Kaplan-Meier survival curves of cumulative (cum) overall survival according to the log rank test. The overall survival rate of ovarian cancer patients with high TCF12 expression (green line) was significantly lower than that of patients with low or no expression (blue line).

quantified using ImageJ software.

2.6. Cell proliferation assay

Cell proliferation was detected using the MTT method (Promega, USA). A total of 1.5×10^3 cells/well were plated into a 96-well plate and grown for 24 h. the Cells were treated as indicated above. After treatment for 24 h, 48 h and 72 h, 10 μl/well of MTT was added, and the cells were incubated at 37 °C for 4 h, protected from light. Then, 150 μl/well DMSO was added and incubated at 37 °C for 10 min. Last, the fluorescence intensity of each well was measured at 570 nm using a microplate reader (BioTek, USA).

2.7. Wound-healing assay

Cell migration was assessed using the wound-healing assay. Briefly, 1×10^5 cells/well were plated and treated as indicated above in a 6-well plate. A wound was made through confluent monolayer cells with a pipette tip after treatment for 4 h. At 0, 24, 48, or 72 h, cells were washed with PBS, and photos were taken to monitor cell migration.

2.8. Transwell assay

A transwell migration assay was used to detect cell invasion. In total, 2×10^5 cells/well were seeded onto a 24-well plate, grown for 24 h, and then treated for 48 h as described above. the Cells were suspended in DMEM at a density of 5×10^5 cells/ml. Transwell chambers (Corning, USA) were incubated with DMEM for 1 h before treatment. Matrigel (BD Biosciences, USA) was thawed at 37 °C overnight, and 100 μl Matrigel (BD Biosciences, USA) (5 mg/ml diluted in serum-free-cold DMEM media) was added to the upper chamber and incubated at 37 °C for 4 h. Then, 100 μl cell suspensions were added to each upper chamber, and 600 μl conditioned medium (cell supernatant with different treatments for 48 h, as described above) was added to the lower chamber. After incubation for 24 h at 37 °C, the cells on the top membrane surface of the upper chamber were carefully removed using cotton swabs, and cells on the bottom surface were fixed in 10% formaldehyde for 30 s, washed with PBS and stained with 0.5% crystal violet solution for 30 min. After being washed with PBS, cells on the bottom surface of the membrane were examined under a microscope and counted in 3–5 random fields.

2.9. Cell apoptosis assay

The Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, USA) was used to evaluate cell apoptosis. Briefly, 3×10^5 cells/well were plated onto a 6-well plate, grown for 24 h, and treated for 48 h as described above. Then, the cells were washed with PBS, trypsinized, transferred into centrifuge tubes and centrifuged for 5 min at 1000 rpm. After being washed in PBS, the cells were re-suspended in 195 μl Annexin V-FITC binding buffer (1×), and 5 μl Annexin V-FITC was added and incubated for 10 min at room temperature, protected from light. Subsequently, the cells were centrifuged for 5 min at 1000 rpm, re-suspended using 190 μl Annexin V-FITC binding buffer (1×), and then 10 μl Propidium Iodide (PI) was added. Last, the cells were analyzed using a Flow Cytometer (BD Biosciences, USA).

2.10. Statistical analysis

All experiments in this study were performed three independent times. The data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS18.0 software. Comparisons between two groups were performed by Student's *t*-test. Significance between multiple groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The survival curve was analyzed using the Kaplan-Meier method. All *P*-values are based on two-tailed statistical analysis. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. TCF12 overexpression in patients with ovarian cancer

To explore TCF12 expression in ovarian cancer, 27 cases of fresh ovarian cancer and paired normal ovary tissues were used to determine the TCF12 expression profiles in patients with ovarian cancer using RT-qPCR. The results showed that the mRNA levels of TCF12 were high expressed in ovarian tumor tissues compared with the levels in normal ovary tissues ($P < 0.01$) (Fig. 1). Furthermore, ovarian cancer cell

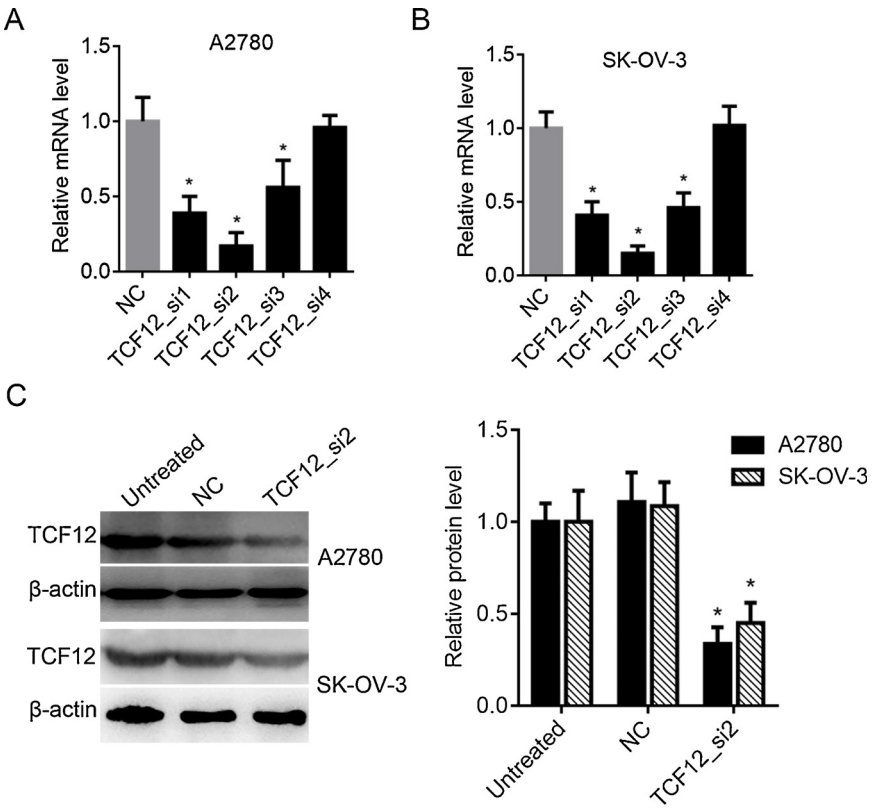


Fig. 4. TCF12 expression inhibited by siRNAs in ovarian cancer cells. **A**. The mRNA levels of TCF12 inhibited by siRNAs in A2780 cells. **B**. The mRNA levels of TCF12 inhibited by siRNAs in SK-OV-3 cells. **C**. The protein levels of TCF12 inhibited by siRNA both in A2780 and SK-OV-3 cells. *$P < 0.05$, compared with NC or untreated cells ($P < 0.05$).

Abbreviations: TCF12, transcription factor 12; siRNA, small interfering RNA; si, small interfering; NC, negative control.

lines were also used to confirm TCF12 expression, and the results also showed that TCF12 mRNA levels were higher in A2780 and SK-OV-3 ovarian cancer cells than in normal IOSE80 ovarian epithelial cells ($P < 0.05$) (Fig. 1**B**).

3.2. Correlation of TCF12 expression and clinicopathological factors in ovarian cancer

A total of 116 cases of ovarian cancer tissue samples in tissue microarray sections were used for TCF12 expression analysis by immunohistochemical staining. The results showed 62.07% (72/116) moderate or strong TCF12 staining in ovarian cancer tissues, 37.93% (44/116) weak staining (Fig. 2 & Table 2), and negative staining in control normal ovarian tissues. Thus, TCF12 expression was higher in cancer tissues than in normal tissues. TCF12 expression was associated with clinicopathological factors in patients with ovarian cancer (Table 2). We found significant differences between positive TCF12 expression and histological grade ($P = 0.020$) and metastasis ($P = 0.002$). In total, 116 patients were used to perform the survival analysis, and the Kaplan-Meier survival curve showed that ovarian cancer patients who presented with high TCF12 expression had poorer overall survival rates than patients who presented with low expression (Fig. 3) ($P < 0.01$).

3.3. siRNAs inhibited TCF12 in ovarian cancer cells

To further analyze TCF12 expression in ovarian cancer cells, predesigned siRNAs (TCF12_si1, TCF12_si2, TCF12_si3 and TCF12_si4) were used to knockdown TCF12 levels. The results showed that TCF12 mRNA levels were significantly decreased by TCF12_si1, TCF12_si2 and TCF12_si3, especially by TCF12_si2, both in A2780 and SK-OV-3 cells compared with NC untreated cells ($P < 0.05$) (Fig. 4**A** & **B**). In addition, the protein levels of TCF12 were also significantly inhibited by TCF12_si2 in A2780 and SK-OV-3 cells (Fig. 4**C**). TCF12_si2 was chosen for a more detailed functional study.

3.4. Inhibitory effect of TCF12 targeted-siRNA on ovarian cancer cell growth

An MTT assay was used to investigate the inhibitory effects of TCF12 targeted-siRNA on ovarian cancer cell growth. TCF12 targeted-siRNA (TCF12_si2) clearly inhibited the growth of A2780 and SK-OV-3 cells at 48 and 72 h compared with NC treated cells ($P < 0.05$) (Fig. 5**A** & **B**).

3.5. Inhibitory effect of TCF12 targeted-siRNA on ovarian cancer cell migration and invasion

A wound-healing assay was used to assess the inhibitory effect of TCF12 targeted-siRNA (TCF12_si2) on ovarian cancer cell migration, and the transwell migration assay was used to evaluate the inhibitory effect of TCF12_si2 on ovarian cancer cell invasion. The results showed that TCF12_si2 treatment significantly decreased A2780 and SK-OV-3 cell migration and invasion versus cells treated with NC siRNA ($P < 0.05$) (Fig. 5**C** & **D**).

3.6. Inhibitory effect of TCF12 targeted-siRNA on ovarian cancer cell apoptosis

Annexin-V-FITC/Propidium Iodide flow cytometry was used to evaluate the inhibitory effect of TCF12 targeted-siRNA (TCF12_si2) on ovarian cancer cell apoptosis. Treatment with TCF12_si2 for 48 h significantly increased apoptosis in both A2780 and SK-OV-3 cells versus cells treated with NC siRNA ($P < 0.05$) (Fig. 5**E**).

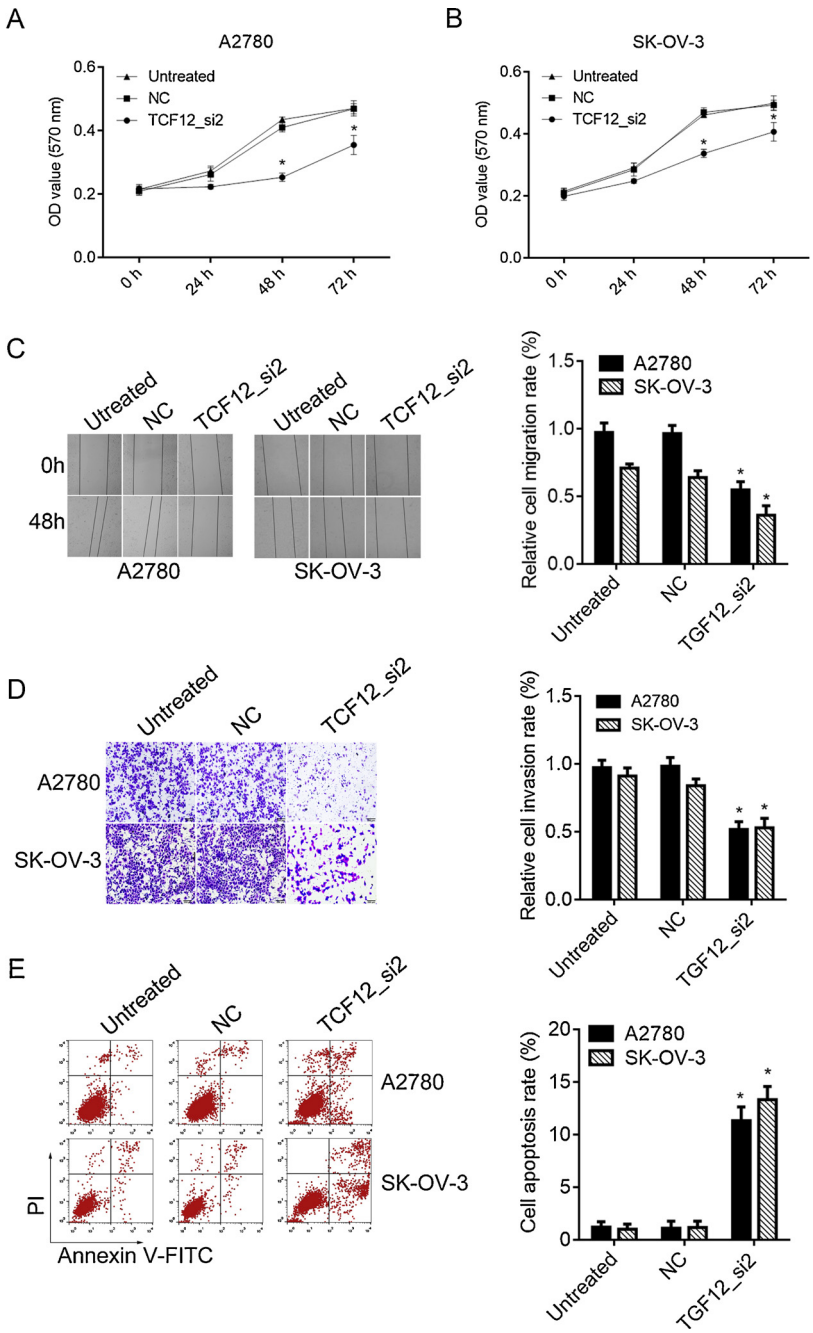


Fig. 5. Growth, migration, invasion and apoptosis were affected by TCF12 downregulation in ovarian cancer cells. **A**. Effect of TCF12 inhibition by siRNA on the growth of A2780 cells detected by the MTT assay. **B**. Effect of TCF12 inhibited by siRNA on the growth of SK-OV-3 cells detected by the MTT assay. **C**. Effect of TCF12 inhibition on ovarian cancer cell migration detected by the wound-healing assay. **D**. Effect of TCF12 inhibition on ovarian cancer cell invasion detected by the transwell migration assay. **E**. Effect of TCF12 inhibition on the apoptosis of ovarian cancer cells detected by Annexin V-FITC/PI staining and FCM. *$P < 0.05$, compared with NC-treated cells.

Abbreviations: TCF12, transcription factor 12; siRNA, small interfering RNA; PI, Propidium Iodide; FCM, flow cytometry; si, small interfering, NC, negative control.

4. Discussion

Ovarian cancer is a malignant tumor, and no early diagnosis or effective therapy is currently available. The bHLH transcription factor TCF12 mediates transcription by forming homo- or heterodimers with other bHLH transcription factors and is highly expressed in some human cancers [16–22], but the mechanism of TCF12 in cancers remains poorly understood. Tang X et al. reported that TCF12 was highly expressed in breast cancer patients and was correlated with a poor prognosis [18]. Lee CC et al. reported that TCF12 was overexpressed in patients with colorectal cancer (CRC), as shown by microarray analysis, and its overexpression was significantly correlated with CRC metastasis [19]. Chen WS et al. found that CRC patients with TCF12 overexpression exhibited a significantly higher rate of metastatic occurrence [20]. He J et al. indicated that TCF12 expression was correlated with poorer overall survival in patients with gallbladder cancer [22]. Nevertheless, the roles of TCF12 in ovarian cancer and other cancers are still obscure. In this study, we first found that TCF12 was highly expressed in patients with ovarian cancer, as detected by RT-qPCR, and immunohistochemical staining showed that TCF12 expression was higher in cancer tissues or cells than in normal tissues or cells (Figs. 1 and 2) and that TCF12 overexpression in ovarian cancer was associated with histological grade and metastasis (Table 2). Kaplan-Meier survival curves indicated that ovarian cancer patients who presented with high TCF12 expression had poor overall survival rates (Fig. 3).

Abnormal TCF12 expression in CRC cells facilitated cell migration and invasion, and ectopic TCF12 expression in CRC cells was associated with cellular epithelial-mesenchymal transition (EMT) [19].

Additionally, Chen WS et al. also indicated that CD91/IKK/NF-κB signaling cascade was involved in secreted heat shock protein 90α (HSP90α)-induced TCF12 expression, leading to E-cadherin downregulation and enhanced CRC cell migration/invasion [20]. Decreased TCF12 expression could suppress the invasion and metastasis of breast cancer cells [18]. RNA interfering (RNAi) technology is an effective strategy for abnormal gene downregulation in mammalian cells, and many small interfering RNA (siRNA) drugs have been tested in clinical trials for treating difficult miscellaneous diseases, especially cancers. Here, we designed and screened effective TCF12 targeted siRNAs for decreasing the expression of endogenous TCF12 in ovarian cells to identify the best siRNA. In ovarian cancer cells A2780 and SK-OV-3, mRNA and protein levels were both significantly reduced by siRNA.

TCF12 has been found to be expressed in skeletal muscle, thymus, B- and T-cells. As a transcription regulator, TCF12 was involved in the initiation of neuronal differentiation and in the proliferation of neural stem and progenitor cells [24]. Additionally, TCF12 could be associated with inhibitor of differentiation-1 (Id1) to suppress E-cadherin expression during the early stage of renal tubular epithelial cell dedifferentiation [25], and it was also shown to downregulate E-cadherin expression in the initiation of EMT in CRC [19]. EMT is considered to be associated with the development of cancers, and cell migration and invasion is an initial and necessary step in tumor metastasis, which is controlled by a series of coordinated cellular and molecular changes, which may include EMT to promote tumor cells separate and migrate from primary tumors [26]. In this study, we also found that TCF12 inhibition contributes to the proliferation, migration and invasion of ovarian cancer cells and promotes apoptosis, perhaps through EMT suppression.

In conclusion, our study first demonstrated that TCF12 is highly expressed and plays a key role in ovarian cancer, thus suggesting that TCF12 may be a valuable prognostic factor in ovarian cancer and that TCF12 could be a therapeutic target for ovarian cancer therapy.

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